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# Rate-limiting roles of the tenase complex of factors VIII and IX in platelet procoagulant activity and formation of platelet-fibrin thrombi under flow

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## ABSTRACT

The importance of factor Xa generation in thrombus formation has not been studied extensively so far. Here, we used mice deficient in either factor VIII or factor IX to determine the role of platelet-stimulated tenase activity in the formation of platelet-fibrin thrombi on collagen. With tissue factor present, deficiency in factor VIII or IX markedly suppressed thrombus growth, fibrin formation and platelet procoagulant activity (phosphatidylserine exposure). In either case, residual fibrin formation was eliminated in the absence of tissue factor. Effects of factor deficiencies were antagonized by supplementation of the missing coagulation factor. In wild-type thrombi generated under flow, phosphatidylserine-exposing platelets bound (activated) factor IX and factor X, whereas factor VIII preferentially co-localized at sites of von Willebrand factor binding. Furthermore, proteolytic activity of the generated activated factor X and thrombin was confined to the sites of phosphatidylserine exposure. With blood from a hemophilia A or B patient, the formation of platelet-fibrin thrombi was greatly delayed and reduced, even in the presence of high concentrations of tissue factor. A direct activated factor X inhibitor, rivaroxaban, added to human blood, suppressed both thrombin and fibrin formation. Together, these data point to a potent enforcement loop in thrombus formation due to factor X activation, subsequent thrombin and fibrin generation, causing activated factor X-mediated stimulation of platelet phosphatidylserine exposure. This implies that the factor VIII/factor IX-dependent stimulation of platelet procoagulant activity is a limiting factor for fibrin formation under flow conditions, even at high tissue factor concentrations.

## Introduction

Platelet thrombus formation is a highly dynamic process that is tightly controlled by the rheological conditions and the activity of blood components at the vascular surface. The formation of a stable thrombus relies on interactions between activated platelets on the one hand, and the coagulation system generating thrombin and fibrin on the other hand.<sup>1-3</sup> *In vivo* mouse models have shown that fibrin formation occurs hand-in-hand with platelet activation in the arterial and venous parts of the circulation.<sup>4,6</sup> Furthermore, *in vivo* and *in vitro* flow studies have indicated that both the extrinsic coagulation pathway – triggered via tissue factor and factor (F) VIIa<sup>7-9</sup> and the intrinsic pathway – triggered via FXII and resulting in FXI and FIX activation<sup>10,11</sup> contribute to the formation of a stable thrombus. Tissue factor becomes exposed after vascular damage,<sup>12</sup> but can also be recruited to a growing thrombus via leukocytes.<sup>13</sup> Taken together, current evidence indicates that dual triggering of the coagulation system is required to generate sufficient amounts of thrombin and fibrin to stabilize the growing thrombus. It is recognized that thrombin and fibrin are generated in a resonance loop with platelet activation via the prothrombinase complex.<sup>14,15</sup> However, the precise mechanism of FX activation via the

tenase complex is not well understood.

Upon stimulation with strong agonists such as collagen and thrombin, platelets expose phosphatidylserine at their surface, as a consequence of Ca<sup>2+</sup>-dependent activation of phospholipid scrambling via anoctamin 6 (*TMEM16F*).<sup>16,17</sup> Exposure of phosphatidylserine stimulates the procoagulant activity of platelets, by enhancing the binding and activation of coagulation factors at the platelet surface.<sup>1,2</sup> A physiological role of phosphatidylserine exposure is evident from the observation that chelation of phosphatidylserine by annexin A5 or a genetic deficiency in anoctamin 6 greatly impairs the formation of thrombi in mouse models *in vivo*.<sup>4,16</sup> Earlier studies showed that phosphatidylserine-exposing platelets stimulate prothrombinase activity, by binding FVa, FXa and prothrombin, resulting in the cleavage of prothrombin into thrombin.<sup>9,15</sup> It has also been demonstrated that phosphatidylserine exposure is a driving force for the formation of fibrin networks on the platelet surface.<sup>18</sup> In analogy, it has frequently been suggested,<sup>19-21</sup> but not directly proven, that platelet phosphatidylserine exposure regulates the tenase complex, *i.e.* the binding of FVIIIa and FIXa to cleave FX into FXa. This is a relevant issue, since FX can also be activated by a direct interaction with the tissue factor/FVIIa complex.<sup>21</sup>

Deficiency of FVIII or FIX in humans with hemophilia A or

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B, respectively, causes a mild to severe bleeding disorder. Early work, particularly using animal models, indicated that in these forms of hemophilia initial platelet plug formation (primary hemostasis) is unaffected, whereas ensuing fibrin formation (secondary hemostasis) is markedly diminished.<sup>22-24</sup> This can be explored in test-tube experiments, suggesting that certain rates of FXa as well as thrombin generation are needed to produce a stable hemostatic platelet-fibrin clot, and that both FVIII and FIX are required for sufficient levels of FXa generation.<sup>25-27</sup> Indeed, in (treated) patients with hemophilia, residual thrombin generation appears to be linked to the remaining levels of FVIII or FIX.<sup>28</sup> Mice deficient in FVIII have appeared to be valuable models for hemophilia A, since they show prolonged bleeding times and delayed thrombus formation under flow *in vivo*.<sup>29-32</sup> Similarly, mice deficient in FIX exhibit a bleeding phenotype and show reduced thrombus formation *in vivo*.<sup>33-35</sup>

In the present study, we aimed to establish the role of FX activation (tenase) via FVIII and FIX in the regulation of a stable, fibrin-platelet thrombus under flow conditions. In order to do so, we measured this process using blood from *F8<sup>-/-</sup>* and *F9<sup>-/-</sup>* mice lacking these factors, and determined the binding of these on the thrombus. The data point to a prominent feed-forward role of locally generated FXa not only in fibrin-clot formation, but also in enhancement of platelet phosphatidylserine exposure.

## Methods

Please see the *Online Supplementary Methods* for additional information.

### Animals

Experiments were approved by the local animal experimental and care committee. *F8<sup>-/-</sup>* and *F9<sup>-/-</sup>* mice and matched wild-type mice came from the Jackson Laboratory and Charles River, respectively. All mice were bred on a C57Bl/6 background. Blood was obtained by aortic puncture under anesthesia (ketamine/xylazine; Eurovet) and collected into 1/10 volume of 129 mM trisodium citrate, as described elsewhere.<sup>36</sup>

### Human blood preparation

Blood was taken from healthy volunteers, a patient with hemophilia A (4% FVIII) and a patient with hemophilia B (5% FIX) after their full informed consent, in accordance with the Declaration of Helsinki. Blood samples were collected by puncture of the median cubital vein into 1/10 volume of 129 mM trisodium citrate.<sup>15</sup> In both patients, the levels of other coagulation factors were in the normal range.

### Whole blood flow chamber experiments

Flow experiments were performed with a parallel-plate transparent flow chamber, containing a coverslip coated with spots of type I Horm collagen (Nycomed Pharma), blocked with Hepes buffer pH 7.45 (137 mM NaCl, 5 mM Hepes, 2.7 mM KCl, 2 mM MgCl<sub>2</sub>, and 0.1% glucose), containing 1% bovine serum albumin.<sup>37</sup> Samples of citrate-anticoagulated blood (400-1500 µL) were made to flow at defined wall-shear rate, as described for murine<sup>38</sup> and human<sup>15</sup> blood. Coagulation was induced by co-perfusion of recalcification buffer using a dual syringe pump system (1:1 mouse: 110 mM NaCl, 13.3 mM CaCl<sub>2</sub>, 6.7 mM MgCl<sub>2</sub>, 0.1% glucose and 0.1% bovine serum albumin; 1:10 human: 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 6.3 mM CaCl<sub>2</sub>, 3.15 mM MgCl<sub>2</sub>, 0.1% glucose and

0.1% bovine serum albumin). Where indicated, blood samples were supplemented with tissue factor (Innovin, 0.1-100 pM; Dade Behring), recombinant human FVIII (2 U/mL), recombinant human FIX (2 U/mL) or rivaroxaban (0.4 or 4 µg/mL, all f.c.). Fluorescent probes, OG488-fibrinogen or AF647-fibrinogen (16 µg/mL, f.c.), were added to the blood prior to perfusion. To detect phosphatidylserine exposure, thrombi on coverslips were post-stained with 0.5 µg/mL FITC-annexin A5. Coagulation factors were detected by post-staining with AF647-labeled antibodies against FVIII(a) or FIX(a) (1:20); control staining was performed with irrelevant AF647-labeled IgG at the same dilution. Where indicated, von Willebrand factor (VWF) was stained with FITC-labeled anti-VWF antibody (20 µg/mL), and FXa binding sites were identified with AF647-labeled FXa (16.5 µg/mL). Randomly captured images were analyzed for surface area coverage of thrombi and fluorescent labels using Metamorph software (Molecular Devices).

### Real-time measurement of thrombin and activated factor X generation in flow chambers

To measure the activities of serine proteases, blood samples were pre-incubated with the fluorogenic thrombin substrate Z-GGR-AMC or FXa substrate Pefluor, each 0.5 mM. Thrombi were generated as above on a collagen surface, after which fluorescence accumulation from cleaved substrate was recorded under stasis, by capturing microscopic images every 30 seconds.<sup>15</sup> Regions of interest with or without thrombi images were analyzed for fluorescence intensity changes with ImageJ software (open source).

### Statistical analysis

The statistical significance of differences between groups was determined with the independent samples t-test, using the Statistical Package for Social Sciences (SPSS 11.0). Data are expressed as mean ± standard error of mean. *P* values < 0.05 are considered statistically significant.

## Results

### Key roles of murine factors VIII and IX in the formation of platelet-fibrin thrombi and platelet procoagulant activity in the presence of tissue factor

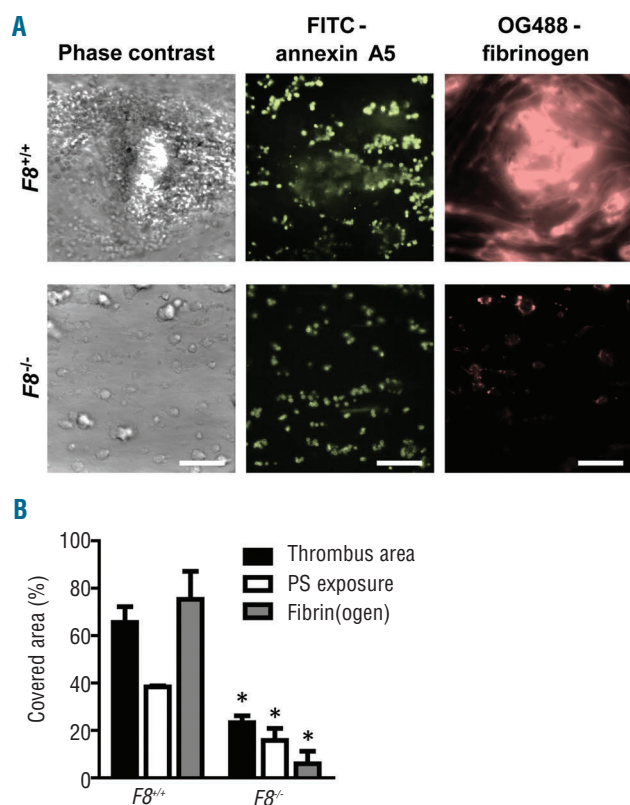
The formation of platelet-fibrin thrombi, triggered via the intrinsic or extrinsic pathway, can be studied efficiently *in vitro* by perfusion of murine or human blood over a collagen surface.<sup>15,38</sup> In order to investigate the role of FVIII in this process, we used citrate-anticoagulated blood from *F8<sup>-/-</sup>* or *F8<sup>+/+</sup>* mice and made this flow during recalcification over collagen at an intermediate wall-shear rate (1000 s<sup>-1</sup>). The blood samples were supplemented with a surplus of 10 pM tissue factor to stimulate the extrinsic pathway. With blood from wild-type *F8<sup>+/+</sup>* mice, this resulted in the formation of large semi-occlusive thrombi after 4 min of perfusion (Figure 1A). The thrombi were composed of aggregated platelets, large patches of phosphatidylserine-exposing platelets (annexin A5 staining) and multiple fibrin fibers (fibrinogen staining). However, blood from *F8<sup>-/-</sup>* mice gave only small-sized platelet thrombi with substantially fewer phosphatidylserine-exposing platelets and limited formation of fibrin (Figure 1A,B). As a consequence, the surface area coverage of *F8<sup>-/-</sup>* platelet-fibrin thrombi was reduced by 64%, when compared to the *F8<sup>+/+</sup>* control condition (*P*=0.002). Fluorescent staining demonstrated a 59% reduction in phosphatidylserine-exposing

platelets (annexin A5) and a 92% reduction in fibrin(ogen) accumulation ( $P < 0.05$ ).

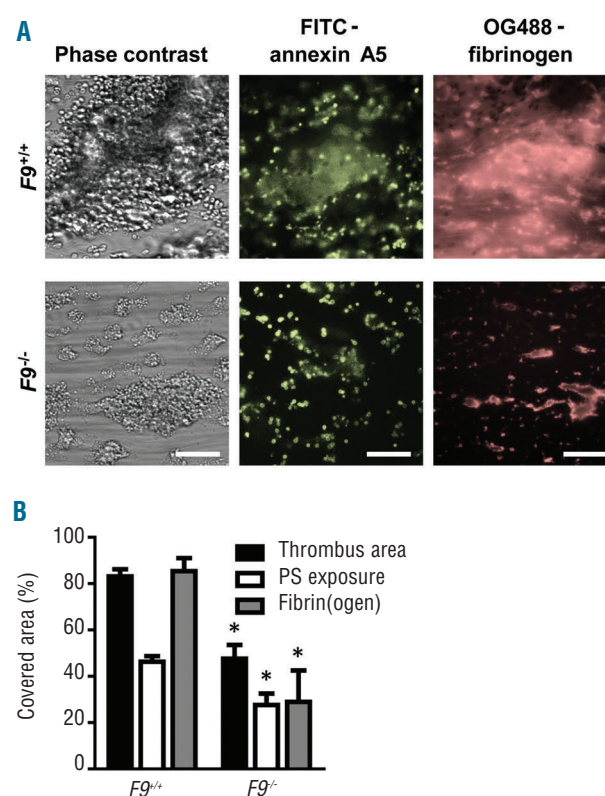
A similar set of flow experiments over collagen was performed with blood from  $F9^{-/-}$  and corresponding  $F9^{+/+}$  mice, again in the presence of tissue factor. Deficiency in FIX also resulted in a major reduction of platelet-fibrin thrombi, as well as decreased phosphatidylserine exposure and fibrin formation (Figure 2A,B). In the case of  $F9^{-/-}$  blood, the surface area covered with thrombi and phosphatidylserine-exposing platelets decreased by 43% and 40%, respectively, compared to the coverage when wild-type blood was used. Interestingly, this reduction was less severe than that observed with FVIII-deficient blood. Regarding thrombus size (surface area coverage) and fibrin formation, the difference between  $F8^{-/-}$  and  $F9^{-/-}$  blood was statistically significant ( $P < 0.005$  and  $P < 0.001$ , respectively). The  $F8$  and  $F9$  wild-type controls did not significantly differ in these parameters. Taken together, these results suggest largely overlapping functions of FVIII and FIX, but a more stringent role of FVIII, in the formation of platelet-fibrin thrombi and in the exposure of phosphatidylserine, under conditions in which exogenous tissue factor is present.

### Maintained roles of murine factors VIII and IX in the formation of platelet-fibrin thrombi without tissue factor

Subsequent flow studies were carried out in the absence of tissue factor to eliminate the contribution of FX activation via tissue factor/FVIIa. We had previously established that in this condition the coagulation process is triggered via collagen-dependent FXII/FXI activation.<sup>38</sup> Flow of wild-type blood resulted in a moderately delayed, but steady formation of platelet-fibrin thrombi, again containing patches of phosphatidylserine-exposing platelets and fibrin fibers (Figure 3B,C). In this condition, flow of  $F8^{-/-}$  or  $F9^{-/-}$  blood (again at a shear rate of  $1000 \text{ s}^{-1}$ ) gave small aggregates still containing procoagulant platelets, whereas fibrin fibers were absent (Figure 3A). More specifically, with blood from  $F8^{-/-}$  mice, the surface area covered by thrombi was reduced by 53%, while phosphatidylserine-exposing platelets were reduced by 37% compared to the wild-type condition ( $P < 0.001$  and  $P < 0.015$ ) (Figure 3B,C). Similar but less stringent reductions were observed with blood from  $F9^{-/-}$  mice. Addition of corn trypsin inhibitor ( $20 \mu\text{g/mL}$ ), to suppress FXII activation, resulted in a further, non-significant reduction of ~20% and 40% in thrombus size for  $F8^{-/-}$  and  $F9^{-/-}$  blood, respectively ( $P > 0.10$ ).



**Figure 1.** Role of murine FVIII in the formation of platelet-fibrin thrombi and procoagulant activity in the presence of tissue factor. Blood from wild-type mice or mice deficient in FVIII was perfused over collagen at a rate of shear  $1000 \text{ s}^{-1}$  under coagulant conditions. Blood samples were pre-incubated with tissue factor ( $10 \text{ pM}$ ) and OG488-fibrinogen. Thrombi were post-stained with FITC-annexin A5 to detect phosphatidylserine (PS) exposure. (A) Representative microscopic phase contrast and fluorescence images after 4 min of flow (bars,  $50 \mu\text{m}$ ). (B) Surface area covered by platelet-fibrin thrombi (black bars), PS-exposing platelets (white bars) and fibrin(ogen) (grey bars) as determined by fluorescence studies. Mean  $\pm$  SEM;  $n = 8-15$ ; \* $P < 0.05$  vs. wild-type.



**Figure 2.** Role of murine FIX in the formation of platelet-fibrin thrombi and procoagulant activity in the presence of tissue factor. Blood from wild-type mice and mice deficient in FIX was perfused over collagen at a rate of shear  $1000 \text{ s}^{-1}$  under coagulant conditions. Blood samples were pre-incubated with tissue factor ( $10 \text{ pM}$ ) and OG488-fibrinogen. Thrombi were then stained with FITC-annexin A5, as in Figure 1. (A) Representative microscopic phase contrast and fluorescence images (bars,  $50 \mu\text{m}$ ). (B) Surface area covered by platelet-fibrin thrombi (black bars), PS-exposing platelets (white bars), and fibrin(ogen) (grey bars) as determined by fluorescence studies. Mean  $\pm$  SEM;  $n = 3-6$ ; \* $P < 0.05$  vs. wild-type.



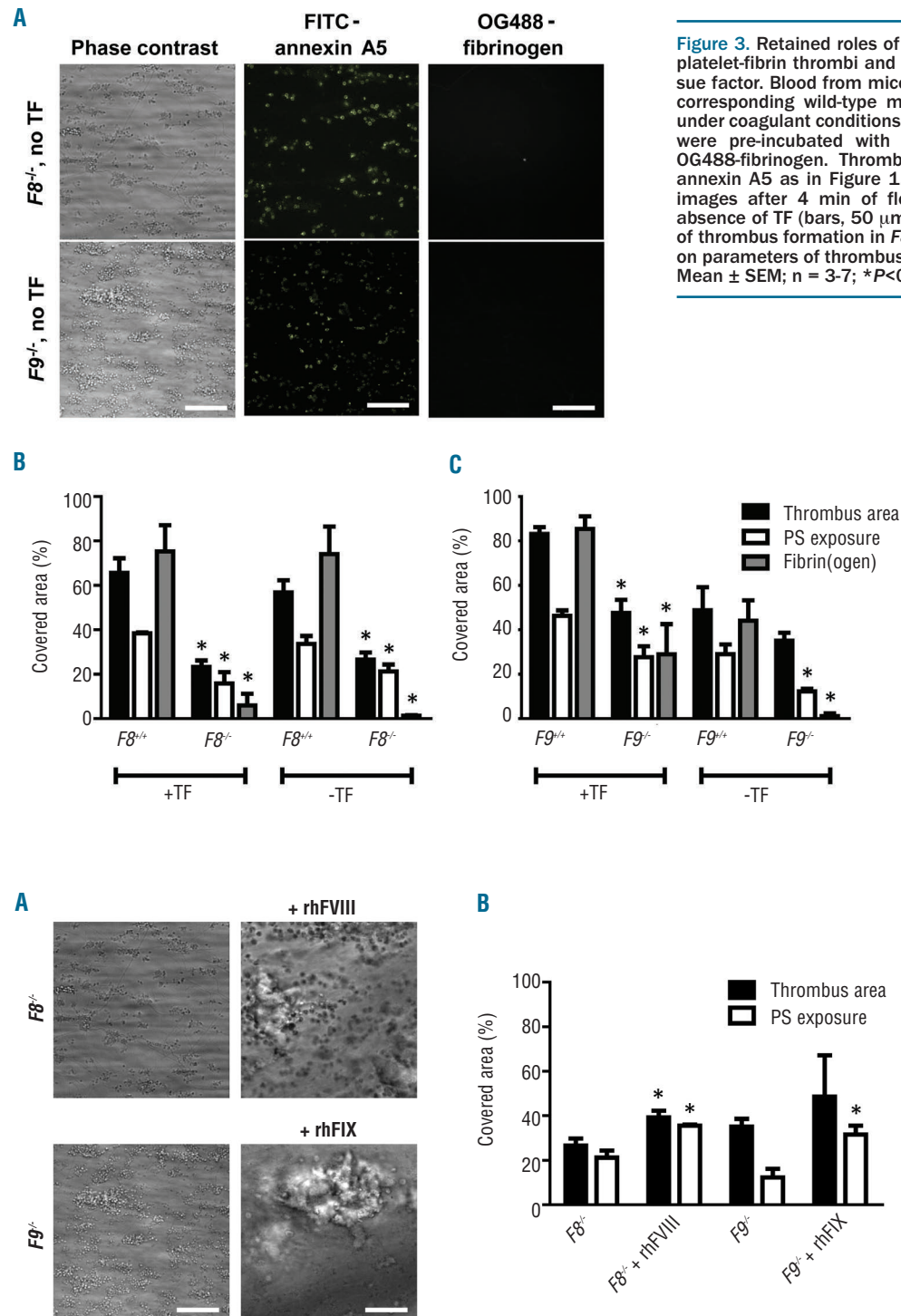
### Improved formation of platelet-fibrin thrombi and platelet procoagulant activity by addition of recombinant human factor VIII or IX

To confirm that the impaired thrombus formation with knockout mice was caused by deficiency in FVIII or FIX, blood samples from  $F8^{-/-}$  or  $F9^{-/-}$  mice were treated with recombinant human FVIII or FIX, respectively. Supplementing blood from  $F8^{-/-}$  mice with recombinant human FVIII resulted in normalization of the clot-formation process and a significant increase in phosphatidylserine-exposing platelets. Similarly, addition of recombinant

human FIX to  $F9^{-/-}$  blood resulted in the formation of large platelet-fibrin clots, which stained positively with FITC-annexin A5 (Figure 4A,B).

### Tenase complex formation and activity on platelet-fibrin thrombi

To determine the localization of the tenase components on the murine thrombi, we first performed staining with fluorescently labeled antibodies against FVIII(a) or FIX(a). Before washing, the fluorescence of each antibody was present around the formed thrombi (Figure 5A). However,



**Figure 3.** Retained roles of FVIII and FIX in the formation of platelet-fibrin thrombi and procoagulant activity without tissue factor. Blood from mice deficient in FVIII or FIX, or from corresponding wild-type mice was perfused over collagen under coagulant conditions. Where indicated, blood samples were pre-incubated with tissue factor (TF, 10 pM) and OG488-fibrinogen. Thrombi were then stained with FITC-annexin A5 as in Figure 1. (A) Representative microscopic images after 4 min of flow of  $F8^{-/-}$  or  $F9^{-/-}$  blood in the absence of TF (bars, 50  $\mu$ m). (B) Effect of TF on parameters of thrombus formation in  $F8^{+/+}$  and  $F8^{-/-}$  mice. (C) Effect of TF on parameters of thrombus formation in  $F9^{+/+}$  and  $F9^{-/-}$  mice. Mean  $\pm$  SEM; n = 3-7; \* $P$ <0.05 vs. corresponding wild-type.

**Figure 4.** Restoration of thrombus formation in factor-deficient mice by supplementation of factor VIII or IX. Blood from  $F8^{-/-}$  or  $F9^{-/-}$  mice was pre-incubated for 10 min with recombinant human FVIII or FIX (2 U/mL), respectively. Samples were perfused for 4 min over collagen at a shear rate of 1000  $s^{-1}$ , as described for Figure 1. Thrombi were then stained with FITC-annexin A5. (A) Representative microscopic phase contrast images (bars, 50  $\mu$ m). (B) Surface area covered by platelet-fibrin thrombi and PS-exposing platelets. Mean  $\pm$  SEM; n = 3.

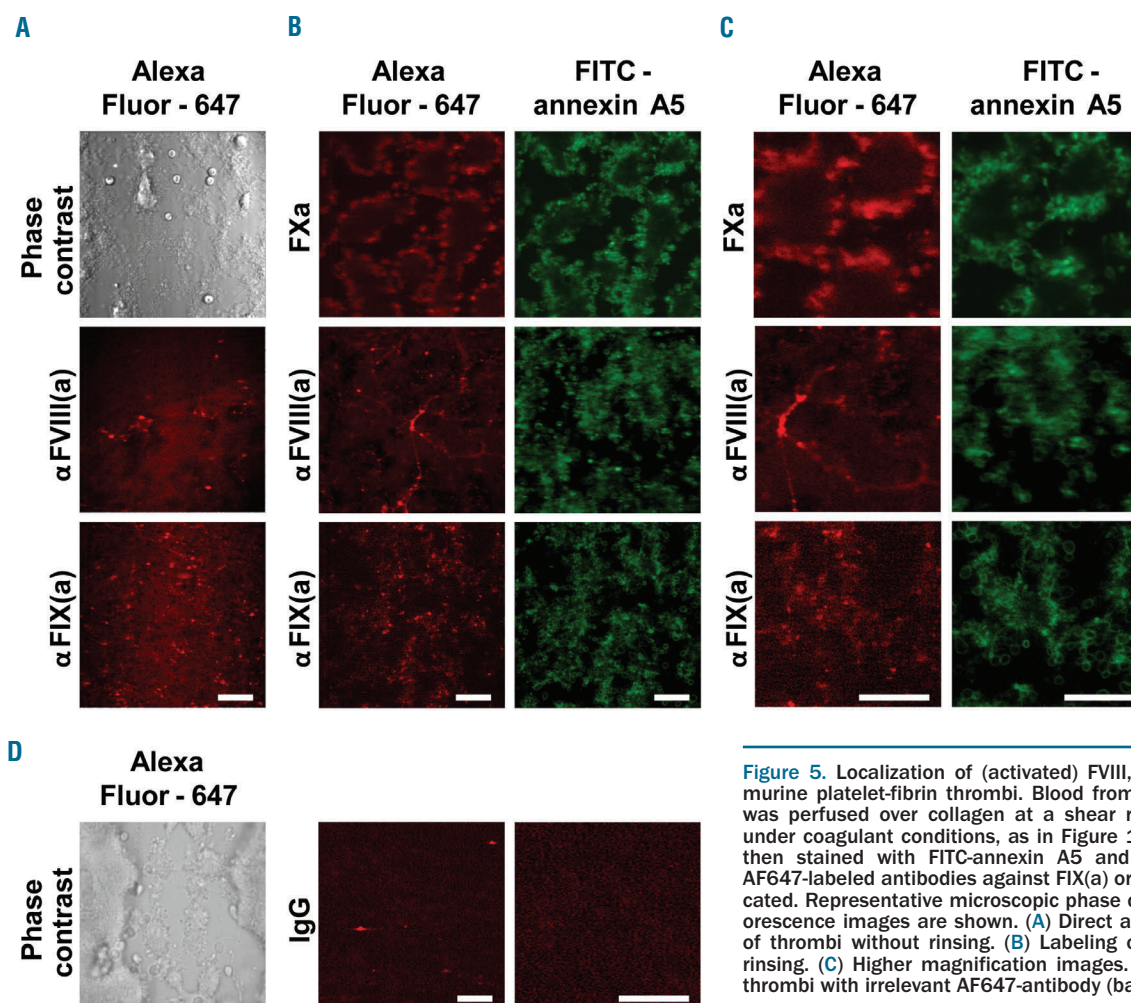
after washing, the staining for FIX(a) was exclusively detected at single platelets in a similar pattern to that seen with labeled annexin A5 staining phosphatidylserine, whereas staining for FVIII(a) was in part concentrated at fibrillar structures (Figure 5B,C). In agreement with previous results,<sup>15</sup> staining of thrombi with the tenase product, labeled FXa, resulted in near complete co-localization with annexin A5-positive platelets. Control experiments with labeled irrelevant antibodies did not result in appreciable fluorescent staining (Figure 5D).

The concentrated staining for FVIII(a) seemed to coincide with earlier staining patterns for VWF, binding to collagen fibers.<sup>39</sup> To investigate this further, we compared the localization of FVIII(a) with VWF and fibrinogen by triple color high-resolution confocal microscopy using fluorescently labeled antibodies against FVIII(a) and VWF as well as labeled fibrinogen (Online Supplementary Figure S1). Image inspection and overlap analysis indicated marked co-localization of FVIII(a) with VWF in part on collagen fibers and furthermore on platelet thrombi in a similar labeling pattern as fibrin(ogen). Together, this points to FVIII(a) bound to VWF as a reservoir for tenase complex formation, in contrast to FIX(a) which seems to be bound in particular to phosphatidylserine-exposing platelets in a thrombus.

To assess the main sites of tenase activity further, murine blood was pre-incubated with fluorescence peptide substrates for thrombin or FXa and, once platelet-fibrin thrombi had been formed, fluorescence microscopic images were taken to determine the generation of thrombin and FXa. Image analysis under stasis indicated consistent activity of both thrombin (Figure 6A) and FXa (Figure 6B), occurring with highly similar kinetics, and being most prominent at areas where platelet thrombi were present. With either substrate, areas in between thrombi showed limited accumulation of fluorescence, most likely due to substrate diffusion from neighboring areas. No fluorescence was seen at microscopic spots where no platelets (no collagen) were present (*data not shown*).

#### **Roles of human intrinsic and extrinsic coagulation pathways in the formation of platelet-fibrin thrombi under flow**

Also with human blood, in the absence of added tissue factor, fibrin clot formation on collagen under flow is known to be initiated by the intrinsic coagulation pathway.<sup>38</sup> To determine the role of the extrinsic pathway herein, we performed perfusion experiments over collagen using whole blood with a range of tissue factor concentrations. Notably, platelet deposition over time was not



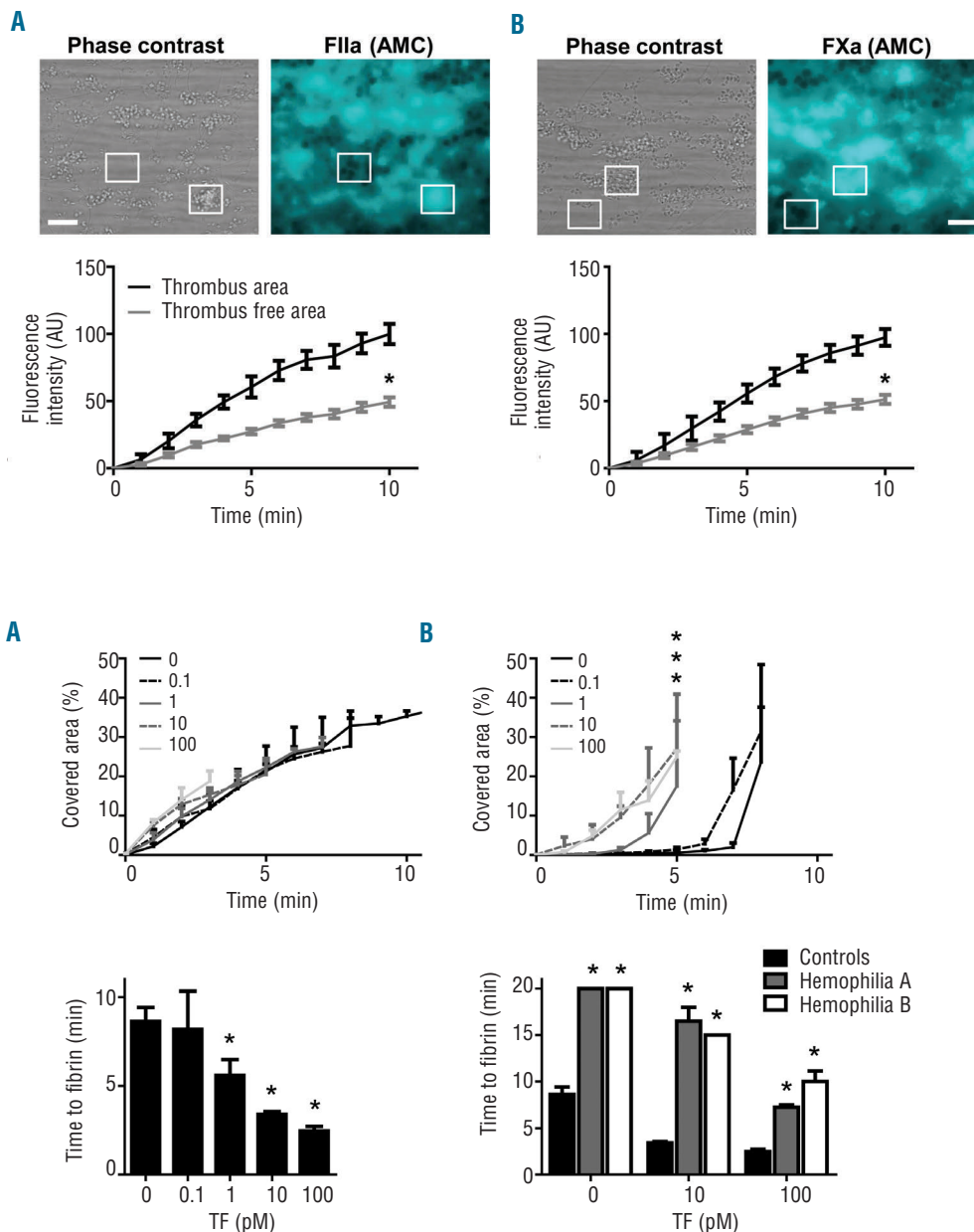
**Figure 5.** Localization of (activated) FVIII, FIX and FX on murine platelet-fibrin thrombi. Blood from wild-type mice was perfused over collagen at a shear rate of 1000 s<sup>-1</sup> under coagulant conditions, as in Figure 1. Thrombi were then stained with FITC-annexin A5 and AF647-FXa, or AF647-labeled antibodies against FIX(a) or FVIII(a), as indicated. Representative microscopic phase contrast and fluorescence images are shown. (A) Direct antibody labeling of thrombi without rinsing. (B) Labeling of thrombi after rinsing. (C) Higher magnification images. (D) Labeling of thrombi with irrelevant AF647-antibody (bars, 20 μm).

affected by increasing the tissue factor concentration (Figure 7A), indicating that this primary process is thrombin independent. However, the extent of fibrin formation was significantly enhanced after 4 minutes of perfusion with 1-100 pM tissue factor (Figure 7B). In agreement with this, time to first fibrin formation decreased dose-dependently with 1, 10 and 100 pM of tissue factor ( $P<0.05$ ,  $P<0.001$  and  $P<0.0001$ , respectively) (Figure 7C).

To assess the amplification role of FVIII and FIX at different trigger strengths, we perfused blood samples from patients with hemophilia A (4% FVIII) or hemophilia B (5% FIX) in the presence of increasing doses of tissue factor. With blood from either patient, no fibrin was formed in the absence of tissue factor over a period of 20 minutes, in comparison to the lag time of  $8.6\pm1.9$  minutes for blood from control subjects ( $P<0.0001$ ). Strikingly, with blood samples from both hemophilic patients, time to fibrin for-

mation shortened at 10 and 100 pM tissue factor, but still remained delayed compared to that of control blood (Figure 5D). These results indicate that the direct pathway of tissue factor/FVII(a)-induced coagulation can contribute to platelet-fibrin thrombus formation under flow, and that both FVIII and FIX enhance this process even at considerably high levels of tissue factor.

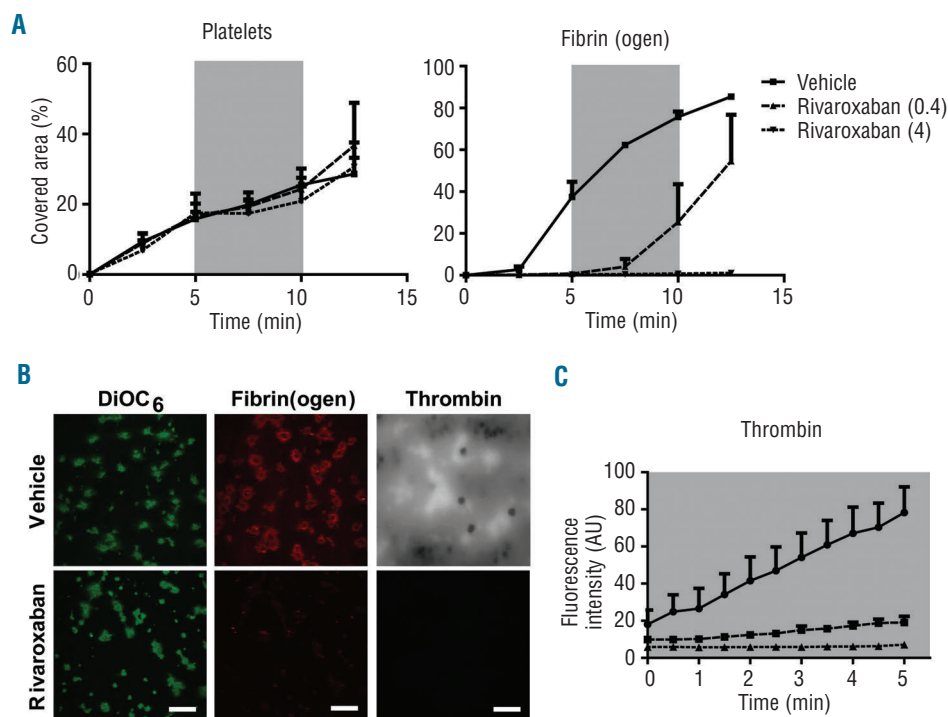
Finally, the sensitivity of this process to anticoagulant treatment was investigated by making human blood, treated with the direct FXa inhibitor rivaroxaban,<sup>40</sup> flow over a collagen/tissue factor surface under coagulant conditions. Rivaroxaban treatment, at 4  $\mu\text{g/mL}$ , did not affect platelet deposition at the collagen surface, but greatly suppressed fibrin accumulation (Figure 8A,B). In agreement with this, rivaroxaban strongly suppressed the cleavage of fluorescence thrombin substrate at the site of platelet thrombi (Figure 8C). This confirmed, also for human



**Figure 6.** Generation of FXa and thrombin on preformed thrombi. Blood from wild-type mice was pre-incubated with (A) thrombin substrate peptide Z-GGR-AMC or (B) FXa substrate peptide Pefluor, and perfused over collagen at a shear rate of  $1000\text{ s}^{-1}$ , as in Figure 1. After 4 min of thrombus formation, a time-series of microscopic fluorescence images was taken under stasis. Representative phase contrast and fluorescence images after 10 min (bars, 20  $\mu\text{m}$ ); and quantification of fluorescence intensity over time of regions of interest containing or not containing thrombi. Mean  $\pm$  SEM;  $n = 8-12$ ,  $*P<0.05$  vs. thrombi.

**Figure 7.** Maintained defective formation of platelet-fibrin thrombi with hemophilic blood at high tissue factor concentrations. Blood from normal control donors or a patient with hemophilia A (HA) or hemophilia B (HB) was perfused over collagen at a shear rate of  $1000\text{ s}^{-1}$  for up to 20 min. Blood samples were pre-incubated with DiOC<sub>6</sub> to label platelets, AF647-fibrinogen and different concentrations of tissue factor (TF, 0-100 pM), as indicated. (A, B) Effect of TF concentration on surface area covered by (A) platelets and (B) fibrin(ogen) with normal blood samples. (C) Effect of TF concentration on time to first fibrin formation. (D) Prolonged time to first fibrin formation with blood from a HA or HB patient. Mean  $\pm$  SEM;  $n = 4-6$ ;  $*P<0.05$  compared to normal controls.





**Figure 8.** Effect of the FXa inhibitor rivaroxaban on the formation of platelet-fibrin thrombi. Human blood was pre-incubated with (A, B) OG488-fibrinogen or (B, C) thrombin substrate peptide Z-GGR-AMC and indicated concentrations of rivaroxaban ( $\mu\text{g/mL}$ ), and then perfused under coagulant conditions over a collagen/tissue factor surface at a shear rate of  $1000 \text{ s}^{-1}$ . After 5 min of flow, the shear rate was reduced to  $150 \text{ s}^{-1}$ , as indicated by the gray areas. Flow was continued at  $1000 \text{ s}^{-1}$  after 5 min. (A) Microscopic images taken at indicated times were quantified for platelet deposition and fibrin(ogen) staining. (B) Representative fluorescence images of DiOC<sub>6</sub>-labeled thrombi, and fibrin(ogen) and accumulated AMC staining. (C) Fluorescence images of AMC fluorescence due to thrombin activity were quantified during 5 min of stasis after an initial 5 min of thrombus formation. Mean  $\pm$  SEM;  $n = 3$ .

thrombi, the presence of a potent enforcement loop of FX activation, thrombin and fibrin generation.

## Discussion

The present data point to key roles of FVIII and FIX in FX activation at the site of a platelet thrombus by supporting: (i) thrombin generation, (ii) thrombus growth and platelet phosphatidylserine exposure, and (iii) fibrin formation at the platelet surface. The likely mechanism is that tenase activity via FVIIIa and FIXa, which is confined to the sites of platelet thrombi, generates FXa that directly catalyzes the conversion of prothrombin into thrombin. The locally produced thrombin fulfills its multiple roles, including fibrinogen cleavage and evoking persistent intracellular signaling in platelets to stimulate phosphatidylserine exposure and enhancement of procoagulant activity.<sup>41</sup>

Early evidence for the assembly of the tenase complex of FVIIIa and FIXa came from experiments using only procoagulant phospholipid membranes.<sup>42,43</sup> Our localization studies of the (activated) coagulation factors on platelet thrombi indicate that both FIX(a) and FXa concentrate on phosphatidylserine-exposing platelets, as expected, because both contain Gla-domains. However, we observed only limited and probably weak binding of FVIII(a) to phosphatidylserine-exposing platelets. Instead, FVIII(a) immuno-activity accumulated at sites of VWF localization, coinciding with either collagen or fibrin(ogen)-labeled platelets in the thrombus. This is in agreement with the established role of VWF as a carrier of FVIII. Given that FVIII is one of the least abundant coagulation factors in plasma, these results suggest that a steady supply of (VWF-bound) FVIII to the procoagulant platelets is needed to ensure continued FXa generation. This sug-

gestion is supported by recent evidence that locally immobilized FVIII increases fibrin clot formation under flow,<sup>44</sup> and that platelet-targeted FVIII can restore hemostasis in hemophilic dogs.<sup>45</sup> Interestingly, engineered FIX variants that stimulate the coagulation process independently of FVIII appeared to reduce bleeding and normalize *in vivo* thrombus formation in hemophilic mice lacking FVIII.<sup>46</sup> This latter observation is compatible with a primary role of FVIII in (soluble?) FIX activation, and the concept that thrombus-localized active FIX is sufficient for FX activation and ensuing thrombin generation.

Based on the observation of low FVIII(a) staining of thrombi before rinsing, and relatively high, concentrated FVIII(a) staining at sites of VWF localization after rinsing, we came to the following hypothesis. Assuming a high affinity of the FVIII(a) antibody, there appears to be low or reversible FVIII(a) binding to procoagulant platelets, i.e. at sites where FIXa is present. Likely, the FVIII(a) can be delivered to those sites from a reservoir of FVIII bound to the VWF on collagen and in the growing platelet thrombus. Earlier studies indicated that binding to VWF has no effect on the kinetics of heavy-chain FVIII activation, but that the cleavage of its light chain enhances the dissociation of FVIII from VWF.<sup>47,48</sup> This is also an attractive mechanism for the presentation of FVIII from its VWF reservoir sites to procoagulant platelets during the thrombus-forming process.

Whereas prothrombinase activity under flow conditions has been studied for 20 years,<sup>49</sup> little has been reported on the regulation of FX activation, i.e. tenase activity, during thrombus formation under flow. Our data indicate that complete deficiency of murine FVIII or FIX leads to a marked impairment of the formation of fibrin-containing thrombi under flow, effects that can be restored by supplementation with human recombinant FVIII or FIX, respec-



tively. Overall, the residual surface area covered by thrombi and fibrin(ogen) appeared to be higher for *F9<sup>-/-</sup>* than for *F8<sup>-/-</sup>* blood in the presence of tissue factor, suggesting that the remaining coagulant activity is higher in the absence of FIX than of FVIII. On the other hand, the data also indicate that without FIX or FVIII, procoagulant platelets and fibrin can still be formed, implying that residual amounts of thrombin are generated with tissue factor present.

In human hemophilia A, the amount of (platelet- or phospholipid-dependent) thrombin generation is considered to be a parameter related to the patient's bleeding phenotype.<sup>28,50</sup> In this context, our findings with hemophilia A and B blood may suggest that local tenase activity at a platelet thrombus is a major regulator of fibrin clot formation and, hence, bleeding cessation. Indeed, even at high tissue factor concentrations, we still observed a prolongation of platelet-dependent fibrin formation under flow conditions. This is even more striking given the residual concentrations of FVIII (4%) and FIX (5%) present in the patients' blood. Jointly, these data point to a limited, supplementary role of tissue factor that still relies on both FVIII and FIX in platelet-fibrin thrombus formation under flow, likely via direct activation of FX.

Other authors have demonstrated that, in mouse models of arterial thrombosis, thrombus formation and fibrin accumulation are greatly impaired in animals deficient in either FVIII or FIX.<sup>30, 35</sup> Flow chamber studies *in vitro* also pointed to diminished fibrin clot formation with blood

from mice lacking FVIII.<sup>32,51</sup> Similar observations have been made in flow assays using blood from patients with FVIII or FIX deficiency under coagulant conditions. It was concluded that platelet deposition and fibrin formation under flow diminished depending on the residual factor activity, with fibrin formation being more sensitive to factor deficiency than platelet deposition.<sup>52-54</sup> The present results extend these findings significantly, by indicating for the first time an enforcing role in the thrombus-forming process of procoagulant, phosphatidylserine-exposing platelets capable of FIX(a) and FXa binding.

We have unraveled a potent enforcement loop in thrombus formation, involving FX activation, subsequent thrombin and fibrin generation, and FXa-mediated stimulation of platelet phosphatidylserine exposure. The FVIII/FIX-dependent stimulation of platelet procoagulant activity seems to be a limiting factor in fibrin clotting under flow conditions.

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Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

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